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<p>(54) Title: BLEACHING ENZYMES</p> <p>(57) Abstract</p> <p>There is provided a bleaching enzyme capable of generating a bleaching chemical and having a high binding affinity for stains present on fabrics. Furthermore, there is provided an enzymatic bleaching composition comprising said bleaching enzyme and a surfactant and a process for bleaching stains present of fabrics.</p>		

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## BLEACHING ENZYMES

### TECHNICAL FIELD

The present invention generally relates to bleaching  
5 enzymes. More in particular, it relates to bleaching enzymes  
capable of generating a bleaching chemical and having a high  
binding affinity for stains present on fabrics. The  
invention also relates to a detergent composition comprising  
10 said enzymes and to a process for bleaching stains present  
on fabrics.

### BACKGROUND AND PRIOR ART

Detergent compositions comprising bleaching enzymes have  
been described in the prior art. For instance, GB-A-2 101  
15 167 (Unilever) discloses an enzymatic bleach composition in  
the form of a hydrogen peroxide-generating system comprising  
a C<sub>1</sub>-C<sub>4</sub> alkanol oxidase and a C<sub>1</sub>-C<sub>4</sub> alkanol. Such enzymatic  
bleach compositions may be used in detergent compositions  
for fabric washing, in which they may provide a low-  
20 temperature enzymatic bleach system. In the wash liquor, the  
alkanol oxidase enzyme catalyses the reaction between  
dissolved molecular oxygen and the alkanol to form an  
aldehyde and hydrogen peroxide. In order to obtain a  
significant bleach effect at low wash temperatures, e.g. at  
25 15-55°C, the hydrogen peroxide must be activated by means of  
a bleach activator. Today, the most commonly used bleach  
activator is tetra-acetyl ethylene diamine (TAED), which  
yields peracetic acid upon reacting with the hydrogen  
peroxide, the peracetic acid being the actual bleaching  
30 agent.

Although this and several other enzymatic bleach systems  
have been proposed, there is still a need for alternative or  
improved enzymatic bleach systems. In particular, the  
35 enzymatic bleach system should be capable of bleaching  
stains which are otherwise difficult to remove, the so-

called "problem stains" such as tea, blackberry juice, or red wine. Such stains would require a significant amount of bleaching for their removal, which might negatively affect the colours of the garment.

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In conventional laundry bleach systems, fabrics are uniformly exposed to the same concentration of bleach, whether "problem stains" are present or not. Moreover, damage to garments (such as the fading of dyes) can be caused by repeated washing with conventional bleach systems, which may contain relatively high concentrations of bleach.

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It is therefore an object of the present invention to provide alternative or improved enzymatic bleach systems which, in particular, should be capable of bleaching stains which are otherwise difficult to remove, and should preferably be more selective in its bleaching action. It is a further object of the present invention to provide an alternative or improved enzymatic bleach process.

20

We have now surprisingly found that it is possible to control the enzymatic bleaching reaction by using the bleaching enzyme according to the invention, which is capable of generating a bleaching chemical and has a high binding affinity for stains present on fabrics. Preferably, the enzyme comprises an enzyme part capable of generating a bleaching chemical, coupled to a reagent having a high binding affinity for stains present on fabrics.

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The new bleaching enzyme is particularly attractive for treating "problem stains" which occur only occasionally, such as tea, red-wine, and blackberry juice. These stains are not present on most garments and when they are present they are likely to be present in different positions than habitual stains such as those found on collars and cuffs. According to the invention, it is possible to optimise the

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in-use concentration of the new bleaching enzyme so that threshold concentrations of bleach are only reached if stain is present and the new bleaching enzyme binds to and accumulates on said stain. When this happens, the high local concentration of enzyme generates a high local concentration of bleach near to the stain and thereby exerts a selective bleaching action where it is required. Therefore, the unstained part of the garment (typically the majority) is not exposed to high levels of bleach and thereby this fabric is protected from bleach-associated damage. Moreover, the next time the same garment has a stain such as blackberry, tea, wine, etc. it is likely to be in a different position on the garment. Therefore, a different position on the garment will be exposed to high levels of bleach. Therefore, problems associated with several washes in conventional bleaching systems, such as dye-fade, will be reduced or eliminated altogether. This is in stark contrast to conventional bleaching systems where all garments are uniformly exposed to high concentrations of bleach, in every wash, regardless of whether problem stains are present or not.

#### DEFINITION OF THE INVENTION

According to a first aspect of the invention, there is provided a bleaching enzyme capable of generating a bleaching chemical and having a high binding affinity for stains present on fabrics. Preferably, the enzyme comprises an enzyme part capable of generating a bleaching chemical, coupled to a reagent having a high binding affinity for stains present on fabrics.

According to a second aspect, there is provided an enzymatic bleaching composition comprising one or more surfactants and the bleaching enzyme according to the invention.

According to a third aspect, there is provided a process for bleaching stains present on fabrics, wherein stained fabrics are contacted with a solution comprising the bleaching enzyme of the invention.

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## DESCRIPTION OF THE INVENTION

### 1. The bleaching enzyme

In its first aspect, the invention relates to a bleaching enzyme which is capable of generating a bleaching chemical and has a high binding affinity for stains present on fabrics. Preferably, the enzyme comprises an enzyme part capable of generating a bleaching chemical which is coupled to a reagent having a high binding affinity for stains present on fabrics.

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#### 1.1 The enzyme part, capable of generating a bleaching chemical.

The bleaching chemical may be enzymatically generated hydrogen peroxide. The enzyme for generating the bleaching chemical or enzymatic hydrogen peroxide-generating system may in principle be chosen from the various enzymatic hydrogen peroxide-generating systems which have been disclosed in the art. For example, one may use an amine oxidase and an amine, an amino acid oxidase and an amino acid, cholesterol oxidase and cholesterol, uric acid oxidase and uric acid or a xanthine oxidase with xanthine. Alternatively, a combination of a C<sub>1</sub>-C<sub>4</sub> alkanol oxidase and a C<sub>1</sub>-C<sub>4</sub> alkanol is used, and especially preferred is the combination of methanol oxidase and ethanol. The methanol oxidase is preferably isolated from a catalase-negative Hansenula polymorpha strain. (see for example EP-A-244 920 (Unilever)). The preferred oxidases are glucose oxidase, galactose oxidase and alcohol oxidase.

A hydrogen peroxide generating enzyme could be used in combination with activators which generate peracetic acid. Such activators are well-known in the art. Examples include tetraacetylenediamine (TAED) and sodium nonanoyl-  
5 oxybenzenesulphonate (SNOBS). These and other related compounds are described in fuller detail by Grime and Clauss in Chemistry & Industry (15 October 1990) 647-653. Alternatively, a transition metal catalyst could be used in combination with a hydrogen peroxide generating enzyme to  
10 increase the bleaching power. Examples of manganese catalysts are described by Hage et al. (1994) Nature 369, 637-639.

Alternatively, the bleaching chemical is hypohalite and the  
15 enzyme part is then a haloperoxidase. Preferred haloperoxidases are chloroperoxidases and the corresponding bleaching chemical is hypochlorite. Especially preferred chloroperoxidases are Vanadium chloroperoxidases, for example from Curvularia inaequalis.

20 Alternatively, peroxidases or laccases may be used. In this case the bleaching molecule is derived from an enhancer molecule that has reacted with the enzyme. Examples of laccase/enhancer systems are given in WO-A-95/01426.  
25 Examples of peroxidase/enhancer systems are given in WO-A-97/11217.

### 1.2 The part having the high binding affinity.

The new bleaching enzyme has a high binding affinity for  
30 stains present on fabrics. It may be that one part of the polypeptide chain of the bleaching enzyme is responsible for the binding affinity, but it is also possible that the enzyme comprises an enzyme part capable of generating a bleaching chemical which is coupled to a reagent having the  
35 high binding affinity for stains present on fabrics. In the first situation, the bleaching enzyme may be a fusion

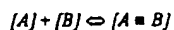
protein comprising two domains which may be coupled by means of a linker. In the second situation, the reagent having the high binding affinity may be covalently coupled to the enzyme part for generating the bleaching chemical, by means of a bi-valent coupling agent such as glutardialdehyde. A full review of chemistries appropriate for coupling two biomolecules is provided in "Bioconjugate techniques" by Greg T. Hermanson, Academic Press Inc (1986). Alternatively, if the reagent having the high binding affinity is a peptide or a protein, it may also be coupled to the enzyme by constructing a fusion protein. In such a construct there would typically be a peptide linker between the binding reagent and the enzyme. An example of a fusion of an enzyme and a binding reagent is described in Ducancel et al. Bio/technology 11, 601-605.

A further embodiment would be for the reagent with a high binding affinity to be a bispecific reagent, comprising one specificity for stain and one for enzyme. Such a reagent could fulfill the requirement of accumulating enzyme on stain either by supplying said reagent together with enzyme as a pre-formed non-covalent complex or by supplying the two separately and allowing them to self-assemble either in the wash liquor or on the stain.

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The novel bleaching enzyme according to the invention is based on the presence of a part having a high binding affinity for stains present on fabrics.

30 The degree of binding of a compound A to another molecule B can be generally expressed by the chemical equilibrium constant  $K_d$  resulting from the following reaction:



The chemical equilibrium constant  $K_d$  is then given by:



$$K_d = \frac{[A] \times [B]}{[A \rightleftharpoons B]}$$

Whether the binding to the stains is specific or not can be judged from the difference between the binding ( $K_d$  value) of the compound to stained (i.e. a material treated so that stain components are bound on), versus the binding to unstained (i.e. untreated) material, or versus the binding to material stained with an unrelated chromophore. For applications in laundry, said material will be a fabric such as cotton or polyester. However, it will usually be more convenient to measure  $K_d$  values and differences in  $K_d$  values on other materials such as a polystyrene microtitre plate or a specialised surface in an analytical biosensor. The difference between the two binding constants should be minimally 10, preferably more than 100, and more preferably, more than 1000. Typically, the compound should bind the stain, or the stained material, with a  $K_d$  lower than  $10^{-4}$  M, preferably lower than  $10^{-6}$  M and could be  $10^{-10}$  M or even less. Higher binding affinities ( $K_d$  of less than  $10^{-5}$  M) and/or a larger difference between coloured substance and background binding would increase the selectivity of the bleaching process. Also, the weight efficiency of the compound in the total detergent composition would be increased and smaller amounts of the compound would be required.

Several classes of compounds can be envisaged which deliver the capability of specific binding to stains one would like to bleach. In the following we will give a number of examples of such compounds having such capabilities, without pretending to be exhaustive.

### 1.2.1. Antibodies.

Antibodies are well known examples of compounds which are capable of binding specifically to compounds against which they were raised. Antibodies can be derived from several  
5 sources. From mice, monoclonal antibodies can be obtained which possess very high binding affinities. From such antibodies, Fab, Fv or scFv fragments, can be prepared which have retained their binding properties. Such antibodies or fragments can be produced through recombinant DNA technology  
10 by microbial fermentation. Well known production hosts for antibodies and their fragments are yeast, moulds or bacteria.

A class of antibodies of particular interest is formed by  
15 the Heavy Chain antibodies as found in Camelidae, like the camel or the llama. The binding domains of these antibodies consist of a single polypeptide fragment, namely the variable region of the heavy chain polypeptide (HC-V). In contrast, in the classic antibodies (murine, human, etc.),  
20 the binding domain consist of two polypeptide chains (the variable regions of the heavy chain ( $V_h$ ) and the light chain ( $V_l$ )). Procedures to obtain heavy chain immunoglobulins from Camelidae, or (functionalized) fragments thereof, have been described in WO-A-94/04678 (Casterman and Hamers) and WO-A-  
25 94/25591 (Unilever and Free University of Brussels).

Alternatively, binding domains can be obtained from the  $V_h$  fragments of classical antibodies by a procedure termed "camelization". Hereby the classical  $V_h$  fragment is  
30 transformed, by substitution of a number of amino acids, into a HC-V-like fragment, whereby its binding properties are retained. This procedure has been described by Riechmann et al. in a number of publications (J. Mol. Biol. (1996) 259, 957-969; Protein. Eng. (1996) 9, 531-537,  
35 Bio/Technology (1995) 13, 475-479). Also HC-V fragments can

be produced through recombinant DNA technology in a number of microbial hosts (bacterial, yeast, mould), as described in WO-A-94/29457 (Unilever).

- 5 Methods for producing fusion proteins that comprise an enzyme and an antibody or that comprise an enzyme and an antibody fragment are already known in the art. One approach is described by Neuberger and Rabbits (EP-A-194 276). A method for producing a fusion protein comprising an enzyme  
10 and an antibody fragment that was derived from an antibody originating in *Camelidae* is described in WO-A-94/25591. A method for producing bispecific antibody fragments is described by Holliger et al. (1993) PNAS 90, 6444-6448.
- 15 A particularly attractive feature of antibody binding behavior is their reported ability to bind to a "family" of structurally-related molecules. For example, in Gani et al. (J. Steroid Biochem. Molec. Biol. 48, 277-282) an antibody is described that was raised against progesterone but also  
20 binds to the structurally-related steroids, pregnanediol, pregnanolone and 6-hydroxy-progesterone. Therefore, using the same approach, antibodies could be isolated that bind to a whole "family" of stain chromophores (such as the polyphenols, porphyrins, or carotenoids as described below).
- 25 A broad action antibody such as this could be used to treat several different stains when coupled to a bleaching enzyme.

#### 1.2.2. Peptides.

- Peptides usually have lower binding affinities to the  
30 substances of interest than antibodies. Nevertheless, the binding properties of carefully selected or designed peptides can be sufficient to deliver the desired selectivity in a oxidation process. A peptide which is capable of binding selectively to a substance which one  
35 would like to oxidise, can for instance be obtained from a

protein which is known to bind to that specific substance.  
An example of such a peptide would be a binding region  
extracted from an antibody raised against that substance.  
Other examples are proline-rich peptides that are known to  
5 bind to the polyphenols in wine.

Alternatively, peptides which bind to such substance can be  
obtained by the use of peptide combinatorial libraries. Such  
a library may contain up to  $10^{10}$  peptides, from which the  
10 peptide with the desired binding properties can be isolated.  
(R.A. Houghten, Trends in Genetics, Vol 9, no 6, 235-239).  
Several embodiments have been described for this procedure  
(J. Scott et al., Science (1990) 249, 386-390; Fodor et al.,  
Science (1991) 251, 767-773; K. Lam et al., Nature (1991)  
15 354, 82-84; R.A. Houghten et al., Nature (1991) 354, 84-86).

Suitable peptides can be produced by organic synthesis,  
using for example the Merrifield procedure (Merrifield  
(1963) J.Am.Chem.Soc. 85, 2149-2154). Alternatively, the  
20 peptides can be produced by recombinant DNA technology in  
microbial hosts (yeast, moulds, bacteria) (K.N. Faber et al.  
(1996) Appl. Microbiol. Biotechnol. 45, 72-79).

### 1.2.3. Peptidomimics.

25 In order to improve the stability and/or binding properties  
of a peptide, the molecule can be modified by the  
incorporation of non-natural amino acids and/or non-natural  
chemical linkages between the amino acids. Such molecules  
are called peptidomimics (H.U. Saragovi et al. (1991)  
30 Bio/Technology 10, 773-778; S. Chen et al. (1992)  
Proc.Natl.Acad. Sci. USA 89, 5872-5876). The production of  
such compounds is restricted to chemical synthesis.

#### 1.2.4. Other organic molecules.

It can be readily envisaged that other molecular structures, which need not be related to proteins, peptides or derivatives thereof, can be found which bind selectively to substances one would like to oxidise with the desired binding properties. For example, certain polymeric RNA molecules which have been shown to bind small synthetic dye molecules (A. Ellington et al. (1990) Nature 346, 818-822). Such binding compounds can be obtained by the combinatorial approach, as described for peptides (L.B. McGown et al. (1995), Analytical Chemistry, 663A-668A).

This approach can also be applied for purely organic compounds which are not polymeric. Combinatorial procedures for synthesis and selection for the desired binding properties have been described for such compounds (Weber et al. (1995) Angew.Chem.Int.Ed.Engl. 34, 2280-2282; G. Lowe (1995), Chemical Society Reviews 24, 309-317; L.A. Thompson et al. (1996) Chem. Rev. 96, 550-600). Once suitable binding compounds have been identified, they can be produced on a larger scale by means of organic synthesis.

#### 1.3 The Stains

For detergents applications, several classes of coloured substances one would like to bleach can be envisaged, in particular coloured substances which may occur as stains on fabrics can be a target. However, it is also important to emphasise that many stains are heterogeneous. Therefore, the substance to be targeted need not itself be coloured providing that it is always present in the mixture of substances that constitute a stain.

Moreover, an important embodiment of the invention is to use a binding compound (refer to 1.2) that binds to several different, but structurally-related, molecules in a class of

"stain substances". This would have the advantage of enabling a single enzyme species to bind (and bleach) several different stains. An example would be to use an antibody which binds to the polyphenols in wine, tea, and  
5 blackberry. Further examples of classes of stain substances are given below:

#### 1.3.1. Porphyrin derived structures.

10 Porphyrin structures, often co-ordinated to a metal, form one class of coloured substances which occur in stains. Examples are heme or haematin in blood stain, chlorophyll as the green substance in plants, e.g. grass or spinach. Another example of a metal-free substance is bilirubin, a  
15 yellow breakdown product of heme.

#### 1.3.2. Tannins, polyphenols

Tannins are polymerised forms of certain classes of polyphenols. Such polyphenols are catechins, leucocyanins, etc. (P. Ribéreau-Gayon, Plant Phenolics, Ed. Oliver & Boyd,  
20 Edinburgh, 1972, pp.169-198). These substances can be conjugated with simple phenols like e.g. gallic acids. These polyphenolic substances occur in tea stains, wine stains, banana stains, peach stains, etc. and are notoriously  
25 difficult to remove.

#### 1.3.3. Carotenoids.

(G.E. Bartley et al. (1995), The Plant Cell 7, 1027-1038). Carotenoids are the coloured substances which occur in tomato (lycopene, red), mango ( $\beta$ -carotene, orange-yellow).  
30 They occur in food stains (tomato) which are also notoriously difficult to remove, especially on coloured fabrics, when the use of chemical bleaching agents is not advised.

#### 1.3.4. Anthocyanins.

(P. Ribéreau-Gayon, Plant Phenolics, Ed. Oliver & Boyd, Edinburgh, 1972, 135-169). These substance are the highly coloured molecules which occur in many fruits and flowers.

- 5 Typical examples, relevant for stains, are berries, but also wine. Anthocyanins have a high diversity in glycosidation patterns.

#### 1.3.5. Maillard reaction products

- 10 Upon heating of mixtures of carbohydrate molecules in the presence of protein/peptide structures, a typical yellow/brown coloured substance arises. These substances occur for example in cooking oil and are difficult to remove from fabrics.

15

#### 2. The Detergent Composition.

- The bleaching enzymes can be used in a detergent composition, specifically suited for stain bleaching purposes, and this constitutes a second aspect of the invention. To that extent, the composition comprises a surfactant and optionally other conventional detergent ingredients. The invention in its second aspect provides an enzymatic detergent composition which comprises from 0.1 - 50 % by weight, based on the total detergent composition, of one or more surfactants. This surfactant system may in turn comprise 0 - 95 % by weight of one or more anionic surfactants and 5 - 100 % by weight of one or more nonionic surfactants. The surfactant system may additionally contain amphoteric or zwitterionic detergent compounds, but this in not normally desired owing to their relatively high cost. The enzymatic detergent composition according to the invention will generally be used as a dilution in water of about 0.05 to 2%.
- 20  
25  
30

- In general, the nonionic and anionic surfactants of the surfactant system may be chosen from the surfactants described "Surface Active Agents" Vol. 1, by Schwartz & Perry, Interscience 1949, Vol. 2 by Schwartz, Perry & Berch, Interscience 1958, in the current edition of "McCutcheon's Emulsifiers and Detergents" published by Manufacturing Confectioners Company or in "Tenside- Taschenbuch", H. Stache, 2nd Edn., Carl Hauser Verlag, 1981.
- 10 Suitable nonionic detergent compounds which may be used include, in particular, the reaction products of compounds having a hydrophobic group and a reactive hydrogen atom, for example, aliphatic alcohols, acids, amides or alkyl phenols with alkylene oxides, especially ethylene oxide either alone  
15 or with propylene oxide. Specific nonionic detergent compounds are C<sub>6</sub>-C<sub>22</sub> alkyl phenol-ethylene oxide condensates, generally 5 to 25 EO, i.e. 5 to 25 units of ethylene oxide per molecule, and the condensation products of aliphatic C<sub>8</sub>-C<sub>18</sub> primary or secondary linear or branched alcohols with  
20 ethylene oxide, generally 5 to 40 EO.

Suitable anionic detergent compounds which may be used are usually water-soluble alkali metal salts of organic sulphates and sulphonates having alkyl radicals containing  
25 from about 8 to about 22 carbon atoms, the term alkyl being used to include the alkyl portion of higher acyl radicals. Examples of suitable synthetic anionic detergent compounds are sodium and potassium alkyl sulphates, especially those obtained by sulphating higher C<sub>8</sub>-C<sub>18</sub> alcohols, produced for  
30 example from tallow or coconut oil, sodium and potassium alkyl C<sub>9</sub>-C<sub>20</sub> benzene sulphonates, particularly sodium linear secondary alkyl C<sub>10</sub>-C<sub>15</sub> benzene sulphonates; and sodium alkyl glyceryl ether sulphates, especially those ethers of the higher alcohols derived from tallow or coconut oil and  
35 synthetic alcohols derived from petroleum. The preferred anionic detergent compounds are sodium C<sub>11</sub>-C<sub>15</sub> alkyl benzene



5 sulphonates and sodium C<sub>12</sub>-C<sub>18</sub> alkyl sulphates. Also applicable are surfactants such as those described in EP-A-328 177 (Unilever), which show resistance to salting-out, the alkyl polyglycoside surfactants described in EP-A-070 074, and alkyl monoglycosides.

10 Preferred surfactant systems are mixtures of anionic with nonionic detergent active materials, in particular the groups and examples of anionic and nonionic surfactants pointed out in EP-A-346 995 (Unilever). Especially preferred is surfactant system which is a mixture of an alkali metal salt of a C<sub>16</sub>-C<sub>18</sub> primary alcohol sulphate together with a C<sub>12</sub>-C<sub>15</sub> primary alcohol 3-7 EO ethoxylate.

15 The nonionic detergent is preferably present in amounts greater than 10%, e.g. 25-90% by weight of the surfactant system. Anionic surfactants can be present for example in amounts in the range from about 5% to about 40% by weight of the surfactant system.

20

The detergent composition may take any suitable physical form, such as a powder, an aqueous or non aqueous liquid, a paste or a gel.

25 The bleaching enzyme used in the present invention can usefully be added to the detergent composition in any suitable form, i.e. the form of a granular composition, a liquid or a slurry of the enzyme, or with carrier material (e.g. as in EP-A-258 068 and the Savinase (TM) and Lipolase  
30 (TM) products of Novo Nordisk). A good way of adding the enzyme to a liquid detergent product is in the form of a slurry containing 0.5 to 50 % by weight of the enzyme in a ethoxylated alcohol nonionic surfactant, such as described in EP-A-450 702 (Unilever).

35

The enzymatic bleaching compositions of the invention comprise about 0.001 to 10 milligrams of active bleaching enzyme per litre. A detergent composition will comprise about 0.001% to 1% of active enzyme (w/w).

5

The enzyme activity can be expressed in units. For example, in the case of glucose oxidase, one unit will oxidise 1  $\mu$ mole of  $\beta$ -D-glucose to D-gluconolactone and  $H_2O_2$  per minute at pH 6.5 at 30°C. The enzyme activity which is added to the enzymatic bleaching composition will be about 2.0 to 4,000 units per litre (of wash liquor).

10

The invention will now be further illustrated in the following, non-limiting Examples.

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In the Figures is:

Figure 1: Two-step protocol. In the two-step protocol, the fabric was rinsed between targetting and the bleaching reaction.

20 Figure 2: One-step protocol. In the one-step protocol, there was not a rinsing step between targetting and the bleaching reaction. Therefore, targetting and bleaching took place in the same medium.

Figure 3: Analysis of BSA-wine immunogen by chromatography on G25 Sephadex.

25 Figure 4: Analysis of PLP/wine mixtures by chromatography on G25 Sephadex.

Figure 5: Investigation of stability of non-covalent complex formed between poly-L-proline and wine pigments.

30 Figure 6: Analysis of antibody binding to red wine.

Figure 7: Cross-reactivity of wine-binding antibodies with other polyphenol-containing foodstuffs that are known to produce problem stains. Cross-reactivity was determined by inhibition analysis.

35

**Example 1.**

Use of a scaled-down model to illustrate the benefits of targeted bio-bleaching.

The following examples 1-5 comprise a scaled-down model system in which a red wine stain on cotton fabric was labelled with a protein antigen for which an antibody is already available. The antibody was covalently coupled to glucose oxidase enzyme using standard procedures, to form an antibody/oxidase conjugate. This conjugate was then applied to the (labelled) wine stain in the presence of glucose. The glucose oxidase enzyme generated  $H_2O_2$  close to the surface of the stain and bleached it. The amount of bleaching was compared with that obtained with the same amount of unconjugated (and therefore untargeted) glucose oxidase. It was also compared with the bleaching obtained by the same amount of glucose oxidase conjugated to a non-specific antibody that did not bind to the protein label. It was also found that particularly good targeting effects could be achieved if the conjugate and glucose were applied in two steps with a rinse in between.

It will be apparent to anyone skilled in the art that methods are available to raise antibodies to small organic molecules. See for example Gani et al. (J. Steroid Biochem. Molec. Biol. 48, 277-282), where antibodies are raised to steroids. Therefore, antibodies could be raised to the constituents of stains by the using the same technical approach. Molecules of interest would include the polyphenols in wine, tea, and blackberry; or the porphyrin rings in blood and grass. Moreover, some antibodies raised against small organic molecules cross-react with other similar structures. For example, in Gani et al. an antibody is described which was raised against progesterone and also binds to the structurally-related steroids, pregnanedione, pregnanolone and 6-hydroxy-progesterone. Therefore, using

the same approach, antibodies could be isolated that bind to the polyphenols in red wine and also to the polyphenols in tea and blackberry. Once such antibodies have been made, their coupling to glucose oxidase and applying to stain would be as below. However, when using these antibodies it would not be necessary to label the stain with protein antigen.

#### Example 2.

##### 10 Labelling stains with protein antigen

White cotton fabric was stained with red wine (Côtes du Rhône, Co-op) and dried in a 37°C incubator. Stained fabric was cut into 1 cm squares and then labelled with a protein antigen, human chorionic gonadotropin (hCG). This was done by applying hCG in the form of a cellulase/hCG conjugate (cellulase binds to cotton fabric). The conjugate was prepared by methods that are well known in the art (see for example "Bioconjugate Techniques" by Greg T. Hermanson, Academic Press (1996)).

20

Cellulase ex. Trichoderma reesei, (Sigma Product number C 8546) was derivatised with N-succinimidyl 3-(2-pyridyldithio) propionate, "SPDP", (Sigma). hCG (Sigma Product number C 5297) was derivatised with S-acetylmercaptosuccinic anhydride, "SAMSA" (Sigma). The two derivatised proteins were then reacted together in pH 6.5 buffer to yield a covalently-coupled conjugate.

The stained fabric squares were labelled with hCG by incubating them in a solution of the cellulase/hCG conjugate. The solution was approximately 10 µg/ml with respect to cellulase and was made up in phosphate buffered saline, PBS, [0.01M Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>-0.15M NaCl, pH 7]. The incubation was for 2 hours at 37°C. Control squares were also prepared that were incubated with cellulase only.

### Example 3.

#### Preparation of antibody / glucose oxidase conjugates.

Antibody / glucose oxidase conjugates were also prepared by  
5 the SAMSA/SPDP method. Antibody was derivatised with SAMSA  
and glucose oxidase was derivatised with SPDP. The two  
derivatised proteins were then reacted together in pH 6.5  
buffer to yield a covalently-coupled conjugate. Two  
different antibodies were used: one that had a specificity  
10 for hCG, "anti-hCG antibody", and one that did not have a  
specificity for hCG, "anti-E3G antibody".

### Example 4.

#### Targeted bleaching of red wine stain - Two-step protocol

15 In the two-step protocol (see Figure 1 for a schematic  
diagram), the fabric squares were rinsed between targeting  
and the bleaching reaction. The bleaching efficiency of the  
anti-hCG / glucose oxidase (GOx) conjugate was compared with  
that of anti-E3G / glucose oxidase conjugate and glucose  
20 oxidase only.

Stained fabric squares, sensitised with either cellulase/hCG  
conjugate or cellulase only, were rinsed once with rinse  
buffer (PBS with 0.5% COCO 6.5 EO added as a surfactant) and  
25 then placed in a 24-well tissue culture plate (Costar). The  
squares were then treated with anti-hCG / glucose oxidase  
conjugate (in rinse buffer), anti-E3G / glucose oxidase  
conjugate (in rinse buffer), glucose oxidase (in rinse  
buffer), or rinse buffer only. The two conjugates had been  
30 normalised for glucose oxidase activity and were applied at  
a concentration equivalent to approximately 15 µg/ml of  
unconjugated enzyme. The fabric squares were left in these  
solutions for 1 hour at room temperature before rinsing them  
3x with rinse buffer. The squares were then treated with  
35 substrate solution (150mM glucose, 30mM NaCl, 0.5% COCO 6.5

EO, 0.1M phosphate (pH 6.5)). The substrate solution was allowed to incubate with the fabric squares for 30 minutes at 37°C. Squares were removed from the substrate solution and dried overnight in a 37°C incubator. Then the amount of stain removed was determined by using a "Color-eye 2020+" spectrophotometer (Macbeth). Fabric squares were read in triplicate and stain removal was recorded as  $\Delta E$  (mean of the three readings) relative to stained, untreated fabric.

It was found that all the fabric squares that had been treated with untargeted glucose oxidase gave similar values for  $\Delta E$ , see Table 1. These different untargeted formats were needed to make sure there were not any artefacts due to non-specific binding between any of the components in the system. Given that all these untargeted formats gave similar readings, it was rigorous to take a mean of these readings, and record this as the mean untargeted enzyme score ( $\Delta E = 20.3$ , see table 1). The fabric squares that had been treated with targeted glucose oxidase gave a value of  $\Delta E = 25.3$ . The mean reading for squares treated with surfactant only was 18.9. (Table 1). Therefore, the untargeted glucose oxidase contributed an improvement of 1.4 units of de-stain above that already achieved by the surfactant in the system. In contrast, the targeted glucose oxidase contributed an improvement of 5.0 units. This means that targeting the glucose oxidase makes it 3.5-fold more effective at removing residual stains, i.e stains that can not be removed by surfactant alone.

### Example 5.

Targeted bleaching of red wine stain - One-step protocol

In the one-step protocol (see Figure 2 for a schematic diagram), there was no rinsing step between targeting and the bleaching reaction. Therefore, targeting and bleaching took place in the same medium. The bleaching efficiency of

the anti-hCG / glucose oxidase conjugate was compared with that of anti-E3G / glucose oxidase and glucose oxidase only.

Samples of fabric (in triplicate) that had been sensitised  
5 with either cellulase/hCG conjugate or cellulase only were added to tubes containing 5 ml of antibody / glucose oxidase conjugate or glucose oxidase only. These were made up in "rinse + substrate buffer" (150mM glucose, 30mM NaCl, 0.5% COCO 6.5 EO, 0.1M Phosphate (pH 6.5)). The tubes were shaken  
10 for 15 minutes at room temperature (to mix thoroughly and to allow the conjugates to bind) and then incubated for 25 minutes at 37°C to allow bleaching to occur.

It is important to note that in the one-step protocol, the  
15 concentration of the conjugates was 100-fold lower than in the two-step protocol (approximately 0.15 µg/ml in terms of glucose oxidase). This reduction in concentration was needed to minimise non-specific binding effects that otherwise occur in the one-step protocol.

20

Reflectances were read in triplicate, as described earlier. The results are shown in Table 2. The targeted enzyme was found to produce stronger bleaching than untargeted enzyme, however, the improvement in bleaching that was attributable  
25 to targeting was less pronounced than that obtained with the two-step protocol.

Table 1: Bleaching of wine stained fabric with two-step protocol

Sensitisation of stained cloth	Bleaching active(s) (applied in presence of surfactant)	Stain removal ( $\Delta E$ )
Cellulase/ hCG conjugate	Anti-hCG/GOx conjugate [targeted system]	25.3
Cellulase	Anti-hCG/GOx conjugate [untargeted system]	21.2
Cellulase/ hCG conjugate	Anti-E3G/GOx conjugate [untargeted system]	21.4
Cellulase	Anti-E3G/GOx conjugate [untargeted system]	20.0
Cellulase/ hCG conjugate	Gox [untargeted system]	19.9
Cellulase	Gox [untargeted system]	19.1
Cellulase/ hCG conjugate	Surfactant only	19.5
Cellulase	Surfactant only	18.3

5

[ $\Delta E$  of white cloth = 30.6]

Mean targeted enzyme score = 25.3

Mean untargeted enzyme score = 20.3

Mean surfactant score = 18.9

10



Table 2: Bleaching of wine stained fabric with one-step protocol

Sensitisation of stained cloth	Bleaching active(s) (applied in presence of surfactant)	Stain removal ( $\Delta E$ )
Cellulase/ hCG conjugate	Anti-hCG/GOx conjugate [targeted system]	22.0
Cellulase	Anti-hCG/GOx conjugate [untargeted system]	20.3
Cellulase/ hCG conjugate	Anti-E3G/GOx conjugate [untargeted system]	19.6
Cellulase	Anti-E3G/GOx conjugate [untargeted system]	20.3
Cellulase/ hCG conjugate	GOx [untargeted system]	19.2
Cellulase	GOx [untargeted system]	19.6
Cellulase/ hCG conjugate	Surfactant only	17.6
Cellulase	Surfactant only	17.3

5 [ $\Delta E$  of white cloth = 30.6]

Mean targeted enzyme score = 22.0

Mean untargeted enzyme score = 19.8

Mean surfactant score = 17.5

**Example 6.**

In the following examples 6-17, red wine is used as the stain to be treated purely to illustrate how the invention may be carried out in practice. The invention is not limited to red wine stains and an analogous approach could be used to raise antibodies to other stains or to chemical constituents of stains.

The constituent chemicals in red wine have molecular masses of a few hundred Daltons (Saucier et al. (1997), Am. J. Enol. Vitic. 48, 370-373) and they are too small to raise an immune response themselves. Therefore, they need to be linked to a larger molecule, such as a protein, to make an "immunogen" i.e. a substance that is capable of generating an immune response. Linking small molecules to proteins to raise an immune response is a well-known technique. For example, Gani et al. (1994) J. Steroid Biochem. Molec. Biol. 48, 277-282 have linked steroids to Bovine Serum Albumin (BSA). In Examples 7-11, two immunogens comprising red wine were prepared and analysed: one was a covalent conjugate of red wine and BSA, the other was a non-covalent complex of red wine and poly-L-proline (PLP).

In Example 15, the wine-binding antibody is coupled to glucose oxidase by using chemical reagents that are marketed for purpose of cross-linking active proteins. However, it would also be possible to achieve the same effect by constructing a fusion protein from a stain-binding antibody and a bleaching enzyme, as described in the text.

**Example 7**Preparation of BSA-wine covalent conjugate.

Red wine (Côtes du Rhône wine (Co-op, U.K.)) was adjusted to neutral pH with dilute NaOH and then 3ml was dispensed into each of 4 tubes. NaIO<sub>4</sub> (Sigma) was freshly made up to 10% in

water and then added drop-wise, in a fume-hood, to the four tubes so that they each contained different concentrations of periodate: 0%, 0.5%, 1% and 2%. The tubes were incubated overnight at room temperature and then each was mixed with  
5 an equal volume of BSA solution (2mg/ml in MES buffer, pH 6.6).  $\text{NaBH}_3\text{CN}$  (Aldrich) was made up to 10% and then added drop-wise, in a fume-hood, to each of the four tubes until the final concentration was equivalent to that of the periodate (i.e. 0%, 0.5%, 1%, or 2% as appropriate). The  
10 mixtures were incubated overnight at room temperature.

#### Example 8

##### Analysis of BSA-wine conjugate.

A ml sample of each of the four preparations was analysed by  
15 gel permeation chromatography, using a PD10 disposable chromatography cartridge (Pharmacia). This cartridge comprises G25 Sephadex. A fresh cartridge was used for each sample. Each cartridge was conditioned in and eluted with phosphate buffered saline or "PBS" [0.01M  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  -  
20 0.15M NaCl, pH 7]. Eluate was analysed by frontal analysis. 1ml fractions were taken and analysed at 518nm (the absorbance maximum for red pigments in wine). In the absence of periodate, there was some red pigment in the high molecular-weight fraction. This was presumably due to the  
25 formation of a non-covalent complex between BSA and wine anthocyanins. When periodate was present at 0.5% or 1%, several-fold more pigment was found in the high molecular-weight fraction. This was due to covalent attachment to albumin. When periodate was present at 2%, very little red  
30 pigment was seen in the high molecular-weight fraction. This was because this sample had developed a heavy precipitate - presumably due to extensive covalent cross-linking of wine pigments and BSA - and therefore could not pass down the column. (Refer to figure 3).

The conjugate prepared with 0.5% periodate was dialysed into 0.15M saline, filtered through an 0.2µm filter (Sartorius) and then kept in the fridge until required for inoculating a rabbit. This was used in preference to the conjugate  
5 prepared with 1% periodate because the latter did not filter very easily, due to the presence of some precipitate.

#### Example 9

#### 10 Manipulation of the interaction between wine polyphenols and PLP.

Proline-rich peptides have been reported in the literature to precipitate polyphenols by multi-site interactions [Siebert et al. (1996) J. Agric. Food Chem. 44, 80-85; Sun and Mattro (1996) Polymer Bulletin 37, 691-698]. In the  
15 present study, it was sought to manipulate this reaction to form soluble complexes that would be suitable for inoculation. Two poly-L-proline (PLP) products were obtained from Sigma: a 7kD product and a 43kD product. The ratio of wine to PLP that was needed to avoid forming a precipitate  
20 was determined by mixing different amounts of wine (pH-neutralised and diluted in water) with different amounts of PLP (made up in PBS), incubating for 1 hour at 37°C, and then examining for formation of precipitate. It was found that under all conditions tested, the 7kD product formed a  
25 precipitate; however, under some conditions, the 43kD protein did not form a precipitate. See Table 3 below.

Table 3

Investigation of precipitate formation when mixing red wine with poly-L-proline (PLP).

Vial	PLP type	PLP concentration	Wine concentration	Visible precipitate? (y/n)
1	43kD	2 mg/ml	50%	n
2	7kD	2 mg/ml	50%	y
3	43kD	2 mg/ml	100%	y
4	7kD	2 mg/ml	100%	y
5	43kD	4 mg/ml	50%	n
6	7kD	4 mg/ml	50%	y

5

#### Example 10

Analysis of PLP/wine mixture for soluble complexes by column chromatography.

A mixture that did form a precipitate, was then investigated to see if it had instead formed a soluble complex (of PLP and wine polyphenols). Wine was pH-neutralised, diluted 1 in 2 in water, and then mixed with an equal volume of 2mg/ml 43kD PLP made up freshly in PBS. The mixture was incubated at 37°C for 1 hour. The presence of complex was determined by gel permeation chromatography followed by frontal analysis at 518nm, as described in Example 8. The chromatogram was compared with that obtained with red wine on its own or red wine mixed with 7kD PLP under the same conditions. In the absence of PLP, the wine pigments bound to the column and did not elute off. However, the sample

mixed with 43kD PLP showed pigment to be present in the high molecular-weight fraction. Therefore, wine pigments and PLP must have been present in the form of a soluble complex. In contrast, the sample containing 7kD PLP showed no pigment in the high molecular weight fraction. This was because this sample had formed a heavy precipitate and therefore could not migrate through the column (refer to Figure 4).

The storage-stability of the complex made with 43kD PLP was then investigated by storing a series of vials (containing said complex) at 37°C and analysing by chromatography at intervals. The storage stability experiment showed that the interaction between wine pigments and PLP was complete in 1 hour and that no detectable dissociation of the complex had occurred even after 20 days at 37°C (Refer to Figure 5). Therefore, the complex was known to be stable at the temperature and for the approximate time-span that would be required for it to function as an immunogen (i.e. in vivo in the rabbit).

## Example 11

### Preparation of PLP/wine complex for inoculation.

An immunogen was prepared for inoculating a rabbit using conditions that were known not to form a precipitate. 43kD PLP was made up to 2mg/ml in PBS. Red wine was adjusted to approximately neutral pH with dilute NaOH and then diluted 1 in 4 in PBS. Then equal volumes of the wine and PLP were mixed together and then incubated for 1 hour at 37°C. The mixture was analysed on a PD10 column (as described in Example 10) to confirm it did indeed contain some soluble complex. It was then filtered through an 0.2µm filter and then stored in the fridge until required for inoculation. Fresh immunogen was prepared for each booster inoculation.

**Example 12**Inoculation of rabbits and recovery of antibody from serum.

One rabbit was inoculated with the BSA-wine conjugate and  
5 one with the polyproline/wine complex, hereafter referred to  
as rabbit 1 and rabbit 2 respectively. The dose given was  
1ml at approximately 1mg/ml protein. The first inoculation  
was given in Freund's complete adjuvant; further  
inoculations (or "boosters") were given approximately once  
10 per month in Freund's incomplete adjuvant.

A pre-bleed was taken from each rabbit prior to inoculation;  
test bleeds were taken for analysis about 10 days after each  
inoculation. Antibody was recovered from sera using a "Hi-  
15 Trap" Protein A cartridge (Pharmacia), according to  
manufacturer's instructions. These antibody preparations or  
"polyclonal antibodies" are hereafter referred to as PCA1 or  
PCA2 according to the rabbit from which they originated.  
Antibody preparations originating from the pre-bleeds are  
20 referred to PCA(pre)1 and PCA(pre)2. Antibody preparations  
were analysed for their ability to bind to constituents of  
red wine and to other polyphenols as described in Examples  
13 and 14. When the analyses (refer to Examples 13 and 14)  
showed that there was a good immune response, a larger serum  
25 sample was prepared from the rabbit and used to make a big  
batch of antibody, again using a Hi-Trap Protein A  
cartridge. This antibody preparation was covalently linked  
to glucose oxidase enzyme as described in Example 15.

**Example 13**Analysis of antibody binding to constituent molecules of red wine.

An Enzyme-linked Immunosorbent assay (ELISA) was assembled as follows: Untreated red wine was passed through an 0.2µm filter (Sartorius) and then dispensed into the wells of microtitre plates (flat-bottomed, high capacity plates from Greiner labortechnik). The plates were then incubated at 37°C overnight to promote adsorption of red wine molecules onto the surface of the wells. Plates that had been thus sensitised with wine were washed 3 x with PBST [PBS + 0.15% tween 20 (Sigma)] and then blocked with 1% chicken egg albumin (Sigma) - made up in PBST and filtered through an 0.2µm filter (Sartorius). The blocking step was for 1 hour at room temperature. Control plates that had not been sensitised with wine were also treated with chicken egg albumin in the same way. Both sensitised and un-sensitised plates were then washed 3 x in PBST before incubating with antibody (PCA1, PCA2, or PCA(pre)1, as defined in example 12). Dilutions of antibody ranged from 10µg/ml to zero and were made up in PBST. Incubation with antibody was for 1 hour at room temperature.

Plates were washed 3 x with PBST and then incubated with goat anti-rabbit/alkaline phosphatase conjugate (Zymed) made up in PBST. The incubation was for 1 hour at room temperature. Plates were washed 3 x with PSBT and then incubated with substrate (1mg/ml pNPP in 1M Diethylamine pH 9.8 + 1mM MgCl<sub>2</sub>). The incubation in substrate was continued until there was sufficient colour development (typically about 20 minutes). Absorbances was read at 450nm in an automated ELISA reader. Both rabbits developed a good immune response to red wine: the antibodies recovered from both rabbits showed several properties that were characteristic



of an efficient binding to wine. First, PCA1 and PCA2 bound to wine-sensitised microtitre plates in a concentration-dependent manner; secondly, PCA1 and PCA2 bound much more readily to wine-sensitised microtitre plates than did antibody recovered from a rabbit that had not (yet) been inoculated [i.e. PCA(pre)1]; thirdly, PCA1 and PCA2 bound more readily to wine-sensitised microtitre plates than they did to un-sensitised plates. Moreover, when antibody was applied at 10µg/ml, it could be seen that in the order of 100-fold more antibody had bound to sensitised plates than to un-sensitised plates. This shows that the antibodies had a much higher affinity for a surface impregnated with red wine than the same surface that had not been treated with wine. Refer to Figure 6.

#### Example 14

##### Analysis of antibody cross-reactivity to other stains.

The ability of the antibody preparations to bind to other structurally-related molecules, the polyphenols that are known to be present in wine and tea, was investigated by inhibition analysis. In this technique, antibody was pre-incubated with potential inhibitors (either wine or blackberry) and then applied to microtitre plates sensitised with wine. If binding to the wine was inhibited, the antibody was known to bind to the inhibitor as well. The experimental details are given below:

Solutions of antibodies PCA1, PCA2, PCA(pre)1 and PCA(pre)2 (as defined in Example 12) were made up to 10µg/ml in twice-strength PBST (i.e. PBS + 0.3% tween). Inhibitors [Tea (typhoo 2g/litre) and blackberry juice (Budgen Ltd U.K.)] were clarified by centrifugation and filtration through a paper filter (Whatman No1). They were then filtered through a 0.2µm filter (Sartorius) and diluted in water to make a

range of concentrations from neat to 1/64. Each dilution of the inhibitors was mixed with each of the antibody solutions and then incubated at room temperature for 30 minutes. After this incubation, the "pre-incubation", the antibody was  
5 applied to a wine-sensitised microtitre plate and evaluated for binding as described in Example 13.

Binding of PCA2 to the wine-sensitised plate was inhibited by pre-incubation with tea; therefore, it was concluded that PCA2 binds to tea. Similarly, it was concluded that PCA1  
10 does not bind to tea and that both PCA1 and PCA2 bind to blackberry. See Figure 7. Therefore, both antibody preparations (PCA1 and PCA2) are broad-action reagents i.e. they can bind to more than one structurally-related compound, or to complex mixtures (such as foodstuffs)  
15 containing such compounds. Such reagents, when coupled to bleach-generating enzymes, can be used to treat more than one stain-type when present on fabric.

The finding that pre-incubation of PCA(pre)1 and PCA(pre)2  
20 with tea or blackberry still did not result in said antibodies binding to the wine-sensitised plates was a useful negative control: it showed that no non-specific binding effects were introduced by the pre-incubation step.

## 25 Example 15

### Chemical coupling of antibody to glucose oxidase enzyme.

Stain-binding antibodies were chemically coupled to glucose oxidase using a method based on those described by Carlsson et al. (1978) Biochem. J. 173, 723-737 and by Hermanson in  
30 Bioconjugate Techniques, page 70-71. Antibody preparations PCA1 and PCA2 were concentrated to 10mg/ml using Centricon

30 tubes (Amicon) and dialysed into 0.1M Phosphate buffer pH 6.5.

0.5ml of each antibody (a total of 5mg) was dispensed into a separate "reactivial" (a glass vial with a magnetic stirrer). 1.48 mg of "SAMSA" [s-acetylmercaptosuccinic anhydride, Sigma A1251] was added to each reactivial [SAMSA was added from a 25mg/ml stock made up in dimethylformamide (Aldrich)] and the solutions were stirred at ambient temperature for 30 minutes. The following solutions were added to each reactivial at 5 minute intervals: 120µl of 0.1M EDTA (after a total time of 35 minutes since adding the SAMSA); 480µl of 0.1M Tris pH 7.0 (after 40 minutes); 480µl of 1M hydroxylamine pH 7.0 (after 45 minutes). The volumes of each mixture were then made up to 2.5ml with 0.1M phosphate buffer (after 50 minutes). Each of the two preparations were "desalted" by passing down a PD10 column (Pharmacia) that had been equilibrated in 0.1M phosphate + 5mM EDTA pH 6.5. A fresh column was used for each preparation. The purpose of the desalting step was to remove unreacted SAMSA and hydroxlyamine. A 3ml protein fraction was collected from each column. Therefore, each fraction contained antibody (derivatised with SAMSA) at approximately 1.6 mg/ml.

25 A stock solution of Glucose oxidase (GOx) [Novo Nordisk. Glucose Oxidase ex. Aspergillus Niger, Product No. COO-05-85-1-del-1-PROs] was concentrated to 5mg/ml and buffer exchanged into 0.1M phosphate pH 7.5 by passing down a PD10 column. 2ml of this preparation (a total of 10 mg) was dispensed into a reactivial. 5 mg of "SPDP" [3-(2-Pyridyldithio)-propionic acid (Sigma P3415)] was dissolved in DMSO (dimethylsulfoxide, Sigma) and added to the GOx solution. The mixture was stirred at ambient temperature for 30 minutes. The preparation was then "desalted" by passing

the solution down a PD 10 column equilibrated in 0.1M phosphate buffer pH 6.5. The purpose of the desalting step was to remove unreacted SPDP and to get the solution to the optimal pH for the conjugation reaction. A 3ml protein  
5 fraction was collected from the column. Therefore, this fraction contained GOx (derivatised with SPDP) at approximately 3.3 mg/ml.

3ml of each of the derivatised antibodies (at 1.6 mg/ml)  
10 were dispensed into separate glass vials. 1.5 ml of derivatised GOx (at 3.3 mg/ml) was added to each of the antibodies. The two mixtures were then placed at 4°C overnight to allow conjugation to take place. These conjugates are hereafter described as PCA1-GOx and PCA2-GOx.  
15 They were stored in the fridge until required. As a control, a non-specific antibody-enzyme conjugate (comprising a rabbit antibody that does not bind to wine pigments but to a completely un-related antigen) was prepared by essentially the same method. This reagent is hereafter described as  
20 PCA(ns)-GOx.

Since chemical conjugation of enzyme can result in a partial loss in activity, it was necessary to determine the enzyme activity per unit volume for each of the conjugates so that  
25 exact equivalence of each could be added in the bleaching experiments. This was done using a colour-generating substrate system. A series of dilutions of each conjugate and of unconjugated GOx was made in 0.1M phosphate buffer pH 6.5. 100µl of each dilution was dispensed into the wells of  
30 a microtitre plate. Then 100µl of substrate solution was added to each well. The Substrate solution was 50mM glucose; 10µg/ml horse radish peroxidase enzyme (Sigma), 10µg/ml tetramethylbenzadine (Sigma); made up in 0.1M phosphate pH 6.5. Colouration in the wells was determined after 3 minutes

by reading the absorbance at 630nm. The GOx activity of each conjugate was approximated by comparing the amount of colour it could generate with that generated by unconjugated GOx.

## 5 Example 16

### Bleaching of wine stain with antibody-enzyme conjugate.

Cotton swatches (2cm x 2cm squares) that had been stained with red wine were rinsed in "wash buffer" i.e. PBS pH 7.2 + 0.075% Co-Co 6.5 EO as a surfactant. 6 of these swatches  
10 (still wet with wash buffer) were added to each of 15 containers of 100ml capacity. 1ml of protein reagent, diluted in wash buffer, was added to each of the 15 containers: containers 1-3 received a dilution of PCA1-GOx; containers 4-6 received a dilution of PCA2-GOx; containers  
15 7-9 received a dilution of PCA(ns)-Gox; containers 10-12 received a dilution of (unconjugated) GOx, containers 13-15 received a reagent blank i.e. wash buffer only. The protein reagents had all been diluted to have the same GOx activity, as described in Example 15. Containers were then incubated  
20 for 10 minutes at ambient temperature. Then 8ml of a glucose solution, made up in wash buffer was added to each container: containers 1,4,7,10,13 received a 1mM glucose solution, containers 2,5,8,11,14 received a 10mM solution of glucose, containers 3,6,9,12,15 received a 50mM solution of  
25 glucose. After addition of glucose solution, the protein reagents were at a final concentration that was equivalent in GOx activity to approximately 60 ng/ml of unconjugated enzyme, for the antibody-enzyme conjugates, this corresponded to approximately 300-400 ng/ml of antibody  
30 protein. The containers were then incubated at 37°C for 1 hour. The swatches were rinsed in distilled water and dried overnight at 37°C. Stain removal was measured by analysing the cotton swatches with a spectrophotometre (Color-eye

7000, Macbeth, New Windsor, NY, USA). Measurements were made relative to an untreated standard (i.e. a cotton swatch that had been stained with red wine but not even rinsed with wash buffer). All six swatches were individually analysed with  
5 the Macbeth instrument. Readings were taken from each side of each swatch and then the mean of these two readings was recorded. The instrument was set up to take readings of  $\Delta E$ ,  $\Delta L$ , and  $\Delta R_{460}$ .

10 In Table 4 below, stain removal data are expressed as  $\Delta E$ : mean  $\Delta E$ -values are recorded as taken from the six  $\Delta E$  readings from the six individual swatches in each container. From these data, units of stain removal above that obtained with wash buffer only were calculated, by subtracting the  
15  $\Delta E$ -value for wash buffer only from the  $\Delta E$ -value for protein + wash buffer. These values are recorded as  $\Delta\Delta E$  in Table 4. This figure can be considered as a measure of the removal of residual stain (i.e. that not removed by the surfactant only) and therefore it is an index of the benefit that is  
20 attributable to the protein reagent: the higher the  $\Delta\Delta E$ , the more effective the protein is at removing stain.

Stain removal above that obtained with wash buffer only was seen in all samples that contained GOx (whether or not the  
25 enzyme was conjugated to antibody). In all cases, increasing the concentration of glucose from 1mM to 10mM improved removal of residual stain (i.e. increased  $\Delta\Delta E$ ) significantly. However, a further increase to 50mM glucose did not produce a further increase in removal of residual  
30 stain, except in the case of (untargeted) GOx. In general, targeting the enzyme to stain by conjugating it to stain-binding antibody, improved the removal of residual stain. For example, when 10mM glucose was used, the targeted enzyme (i.e. that linked to PCA1 or PCA2) gave a 4-fold higher  $\Delta\Delta E$ -

value than the same amount of untargeted enzyme (i.e. either no antibody or non-specific antibody). See Table 4.

Table 4: Bleaching of wine stain from cotton fabric.

5

Treatment*			Stain removal ( $\Delta E$ )	Stain removal above "wash buffer" only ( $\Delta\Delta E$ ).
<u>Container</u>	<u>Protein</u>	<u>Glucose</u>		
1	PCA1-GOx	1mM	16.4	2.0
2	PCA1-GOx	10mM	19.2	4.8
3	PCA1-GOx	50mM	19.0	4.4
<u>Container</u>	<u>Protein</u>	<u>Glucose</u>		
4	PCA2-GOx	1mM	15.8	1.4
5	PCA2-GOx	10mM	19.1	4.7
6	PCA2-GOx	50mM	18.5	3.9
<u>Container</u>	<u>Protein</u>	<u>Glucose</u>		
7	PCA(ns)-GOx	1mM	14.9	0.5
8	PCA(ns)-GOx	10mM	15.7	1.3
9	PCA(ns)-GOx	50mM	15.6	1.0
<u>Container</u>	<u>Protein</u>	<u>Glucose</u>		
10	GOx	1mM	14.6	0.2
11	GOx	10mM	15.5	1.1
12	GOx	50mM	16.4	1.8
<u>Container</u>	<u>Protein</u>	<u>Glucose</u>		
13	None	1mM	14.4	-
14	None	10mM	14.4	-
15	None	50mM	14.6	-

\* Each of the above were applied in "wash buffer" i.e. phosphate buffered saline pH 7.2 containing Co-Co 6.5 EO as a surfactant.

10

Table 5 below records the spread of the data collected. For treatments at 10mM glucose, the  $\Delta E$ ,  $\Delta L$ , and  $\Delta R_{460}$  readings are recorded for all six swatches.

5 Table 5: Repeat values for bleaching of wine stain from cotton fabric

Treatment*	$\Delta E$	$\Delta L$	$\Delta R_{460}$
PCA1-GOx (Container 2)	19.3	13.3	17.3
	18.7	12.0	14.8
	19.8	13.9	17.8
	18.6	12.1	15.1
	18.6	12.0	15.0
	20.3	14.6	19.3
PCA2-GOx (Container 5)	18.5	12.2	15.9
	19.1	13.1	17.1
	18.8	12.6	16.3
	19.5	13.6	17.7
	19.0	13.0	17.1
	19.7	13.9	18.5
PCA(ns)GOx (Container 8)	15.4	7.9	10.9
	15.9	9.0	12.2
	15.8	8.5	11.4
	16.7	10.1	13.8
	15.2	7.4	9.1
	15.3	7.7	9.7
GOx (Container 11)	15.8	8.9	n.d.
	15.1	7.5	n.d.
	15.4	7.8	n.d.
	16.1	9.0	n.d.
	15.7	8.4	11.1
	15.0	7.3	10.1
Reagent blank (Container 14)	14.2	5.8	9.3
	15.0	7.5	11.6
	14.0	5.2	8.2
	14.5	6.7	10.4



	14.5	6.5	10.1
	14.1	5.8	9.4

\* Each of the above were applied in "wash buffer" (i.e. phosphate buffered saline pH 7.2, containing 0.075% Co-Co 6.5 EO as a surfactant) and 10mM glucose.

5 [n.d. = not determined].

Table 5 shows that it does not matter whether  $\Delta E$ ,  $\Delta L$ , or  $\Delta R_{460}$  readings are used as a measure of stain removal: in all cases targeted enzyme (i.e. PCA1-GOx or PCA2-GOx) shows an  
 10 increased stain removal over untargeted enzyme (i.e. PCA(ns)-GOx or GOx only). Furthermore, the six repeat values for each treatment are very similar and therefore there is a high degree of confidence that targeted enzyme really does improve stain removal.

15

#### Example 17

Treating dyed cotton with antibody-enzyme conjugate.

Fabric dyed with sulphur green or sulphol orange were used in these experiments because these dyes are known to be  
 20 sensitive to bleach. The aim of these experiments was to determine if the wine-binding antibodies showed any cross-reactivity to the dye molecules, that could result in targeting the enzyme to the dye and therefore to its bleaching. (Bleaching is usually referred to as "fading" for  
 25 dyes).

Dyed cloth was washed in PBS + 0.075% Coco 6.5 E.O before incubation with either antibody-enzyme conjugate, free GOx or wash buffer only. Glucose was used at 10mM throughout and  
 30 incubation was for 1 hour at 37°C. In all other respects, the procedure was as described in Example 16.

Results of these experiments showed that dye fade with antibody targeted oxidase was not significantly increased over untargeted oxidase. See Tables 6a and 6b below.

Therefore, the antibodies raised to wine show little or no cross-reactivity with the dye molecules. Furthermore, the destain to dye-fade ratio at 10mM glucose is approximately 40. [Compare data in Table 4 with data in Table 6]. This means that the antibody-enzyme conjugate used together with 10mM glucose shows a very high potential for removing stains such as red wine without damaging dyes on fabric.

Table 6a. Dye fade on Sulphol orange

Treatment†	Dye fade ( $\Delta E$ )	Dye fade above "wash buffer" only. ( $\Delta\Delta E$ )
PCA1-GOx	2.5	0.1
PCA2-GOx	2.3	-0.1
PCA(ns)-GOx	2.6	0.2
GOx	2.4	0.0
None	2.4	-

Table 6b. Dye fade on Sulphur green 6

Treatment†	Dye fade ( $\Delta E$ )	Dye fade above "wash buffer" only. ( $\Delta\Delta E$ )
PCA1-GOx	0.96	-0.04
PCA2-GOx	1.2	0.2
PCA(ns)-GOx	1.2	0.2
GOx	1.5	0.5
None	1.0	-

† Each of the above were applied in "wash buffer" (i.e. phosphate buffered saline pH 7.2, containing 0.075% Co-Co 6.5 EO as a surfactant) and 10mM glucose.

CLAIMS

1. Bleaching enzyme capable of generating a bleaching  
chemical and having a high binding affinity for stains  
5 present on fabrics.
2. Enzyme according to claim 1, wherein the enzyme  
comprises an enzyme part capable of generating a bleaching  
chemical which is coupled to a reagent having a high binding  
10 affinity for stains present on fabrics.
3. Enzyme according to any one of the preceding claims,  
wherein the enzyme part is an oxidase and the bleaching  
chemical is hydrogen peroxide.  
15
4. Enzyme according to claim 3, wherein the oxidase is  
selected from the group consisting of glucose oxidase,  
galactose oxidase and alcohol oxidase.
- 20 5. Enzyme according to claim 1, wherein the enzyme part is  
a haloperoxidase and the bleaching chemical is hypohalite.
6. Enzyme according to claim 5, wherein the enzyme part is  
a chloroperoxidase and the bleaching chemical is  
25 hypochlorite.
7. Enzyme according to claim 6, wherein the  
chloroperoxidase is a Vanadium chloroperoxidase.
- 30 8. Enzyme according to claim 7, wherein the Vanadium  
chloroperoxidase is Curvularia inaequalis chloroperoxidase.
9. Enzyme according to anyone of the preceding claims,  
where the enzyme part is a laccase or a peroxidase and the

bleaching molecule is derived from an enhancer molecule that has reacted with the enzyme.

10. Enzyme according to any one of the preceding claims,  
5 wherein the enzyme part is coupled to a reagent having a high binding affinity for porphyrin derived structures, tannins, polyphenols, carotenoids, anthocyanins, maillard reaction products.
- 10 11. Enzyme according to any one of the preceding claims, wherein the enzyme part is coupled to a reagent having a high binding affinity for porphyrin derived structures, tannins, polyphenols, carotenoids, anthocyanins, or maillard reaction products when they are adsorbed onto a surface.
- 15 12. Enzyme according to claim 11 wherein the surface is cotton, polyester, or polyester/cotton fabric.
13. Enzyme according to any one of the preceding claims,  
20 wherein the reagent having a high binding affinity is a protein or a peptide.
14. Enzyme according to any one of the preceding claims, wherein the reagent having a high binding affinity is an  
25 antibody, an antibody fragment, or a derivative thereof.
15. Enzyme according to any one of the preceding claims, which is a fusion protein comprising a bleaching enzyme and all or part of a heavy chain immunoglobulin that was raised  
30 in Camelidae and has a specificity for stain molecules.
16. Enzyme according to any one of the preceding claims, wherein the reagent having a high binding affinity has a chemical equilibrium constant  $K_d$  for the substance of less  
35 than  $10^{-4}$  M, preferably less than  $10^{-6}$  M.

17. Enzyme according to claim 16, wherein the chemical equilibrium constant  $K_a$  is less than  $10^{-7}$  M.
18. A bispecific antibody or antibody fragment or a diabody  
5 or an analogous structure arranged so that one specificity is directed to stains present on fabrics and the other is directed to a bleaching enzyme.
19. A multi-specific antibody or antibody or an analogous  
10 structure arranged so that at least one specificity is directed to stains present on fabrics and the others are directed to one or more bleaching enzymes.
20. An antibody or antibody fragment or derivative thereof  
15 that binds to chemical constituents that are present in tea, blackberry and red wine.
21. Enzymatic bleaching composition comprising one or more  
20 surfactants and a bleaching enzyme according to claims 1-17.
22. Enzymatic bleaching composition comprising one or more  
surfactants, a bleaching enzyme according to claims 1-17  
producing hydrogen peroxide and an activator which generates  
peracetic acid.
- 25 23. Enzymatic bleaching composition comprising one or more  
surfactants, a bleaching enzyme according to claims 1-17  
producing hydrogen peroxide and a transition metal catalyst.
- 30 24. Process for bleaching stains present on fabrics,  
wherein stained fabrics are contacted with a solution  
comprising the bleaching enzyme according to claims 1-17.
- 25 25. Process for bleaching stains present on fabrics,  
35 wherein stained fabrics are contacted with an enzymatic  
bleaching composition according to claims 21-23.

26. An immunogen that comprises polyphenols and poly-L-proline.

\*\*\*\*\*

Fig.1.

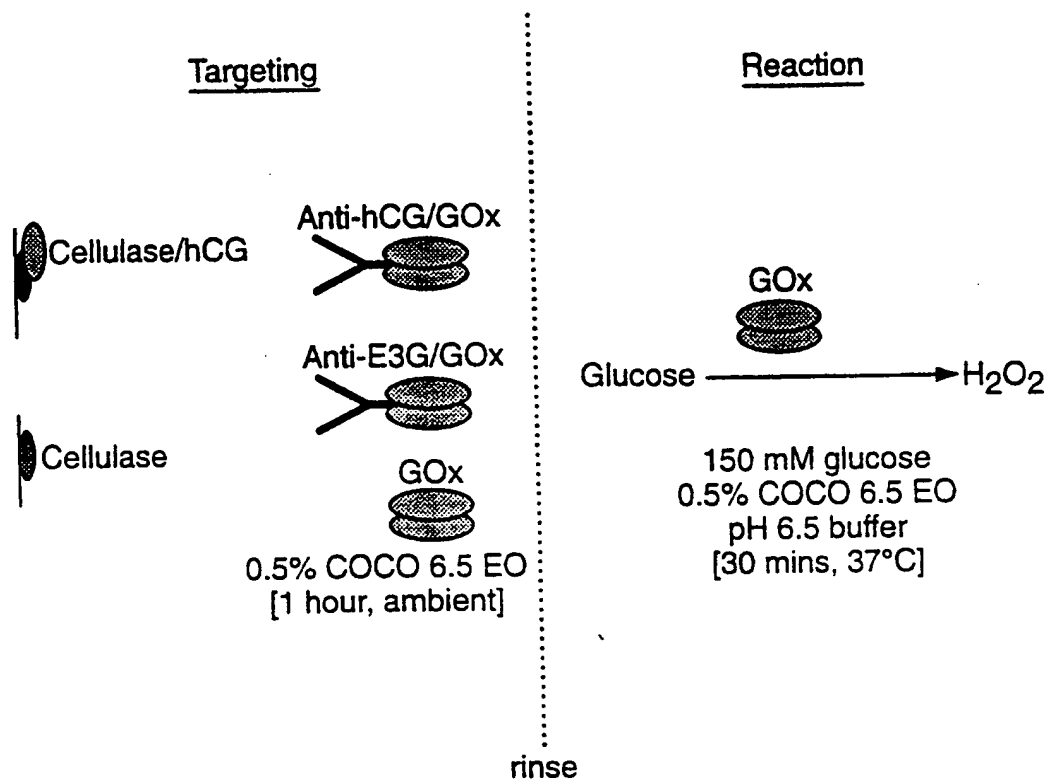


Fig.2.

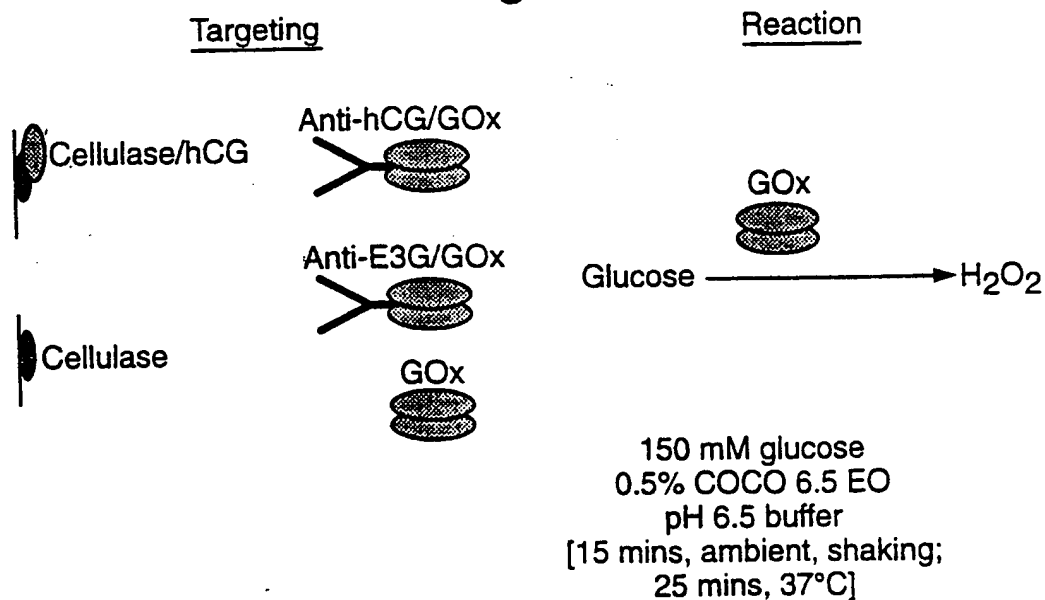


Fig.3.

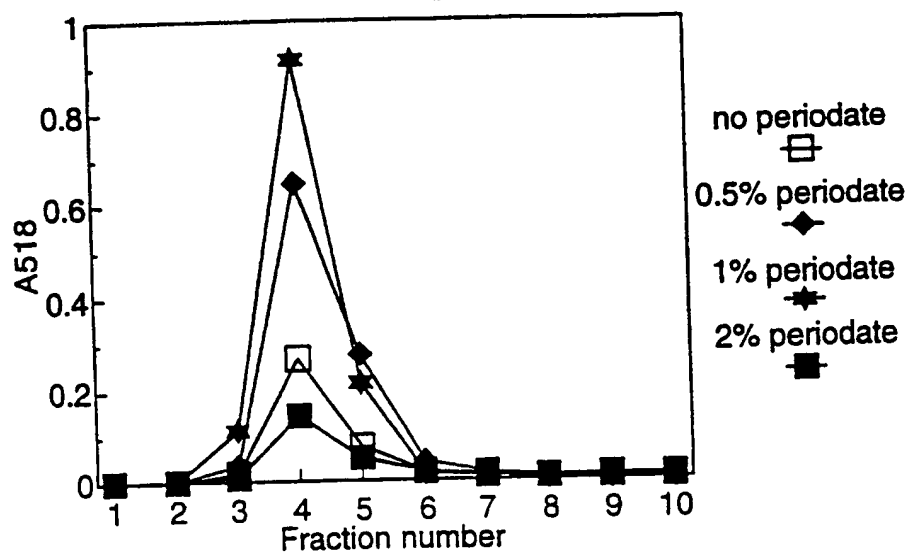


Fig.4.

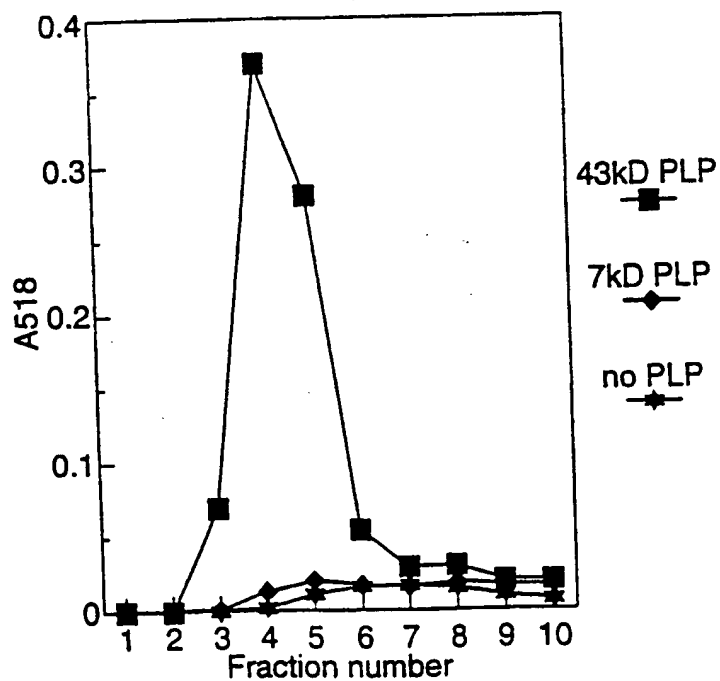




Fig.5.

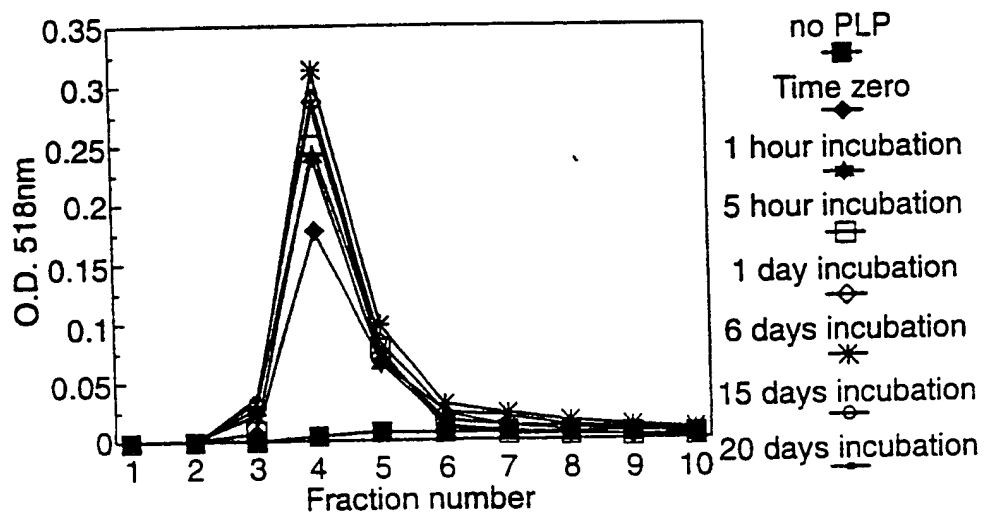


Fig.6.

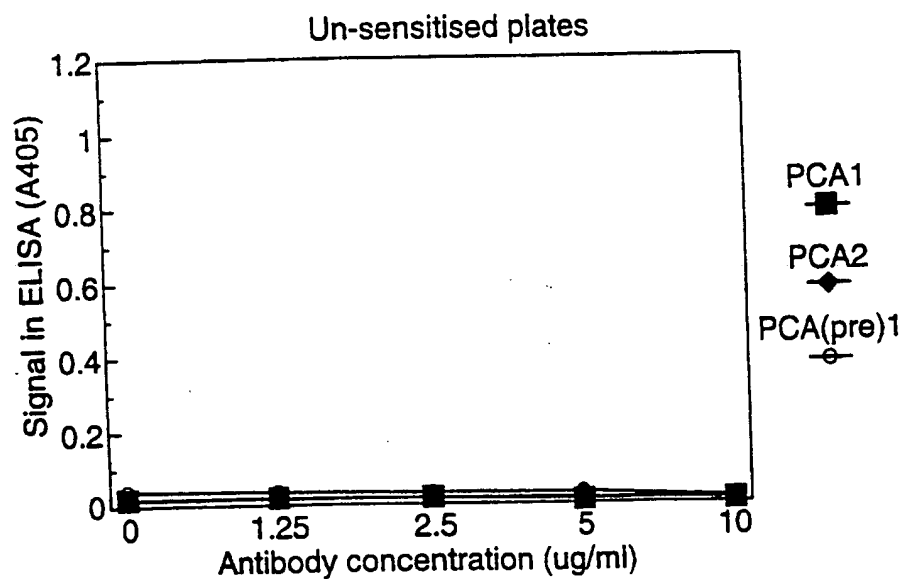
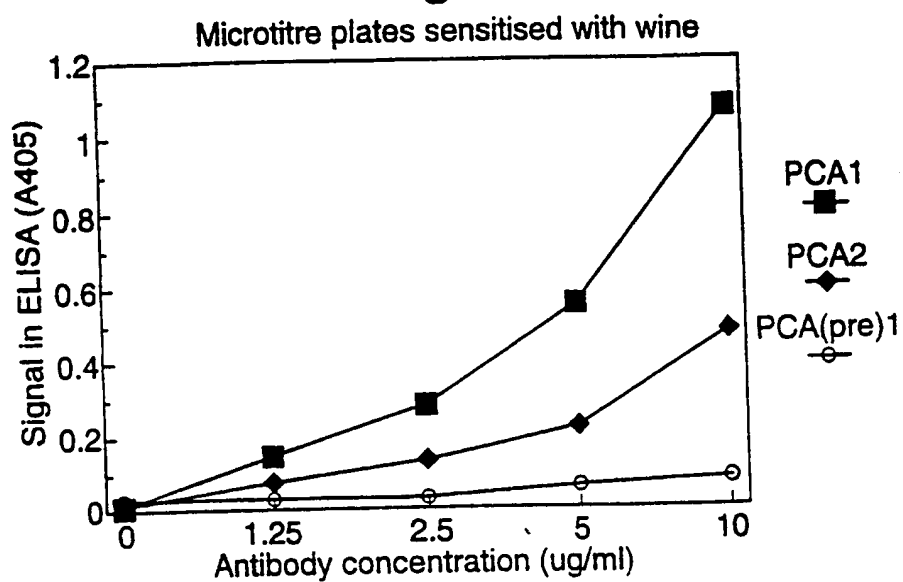
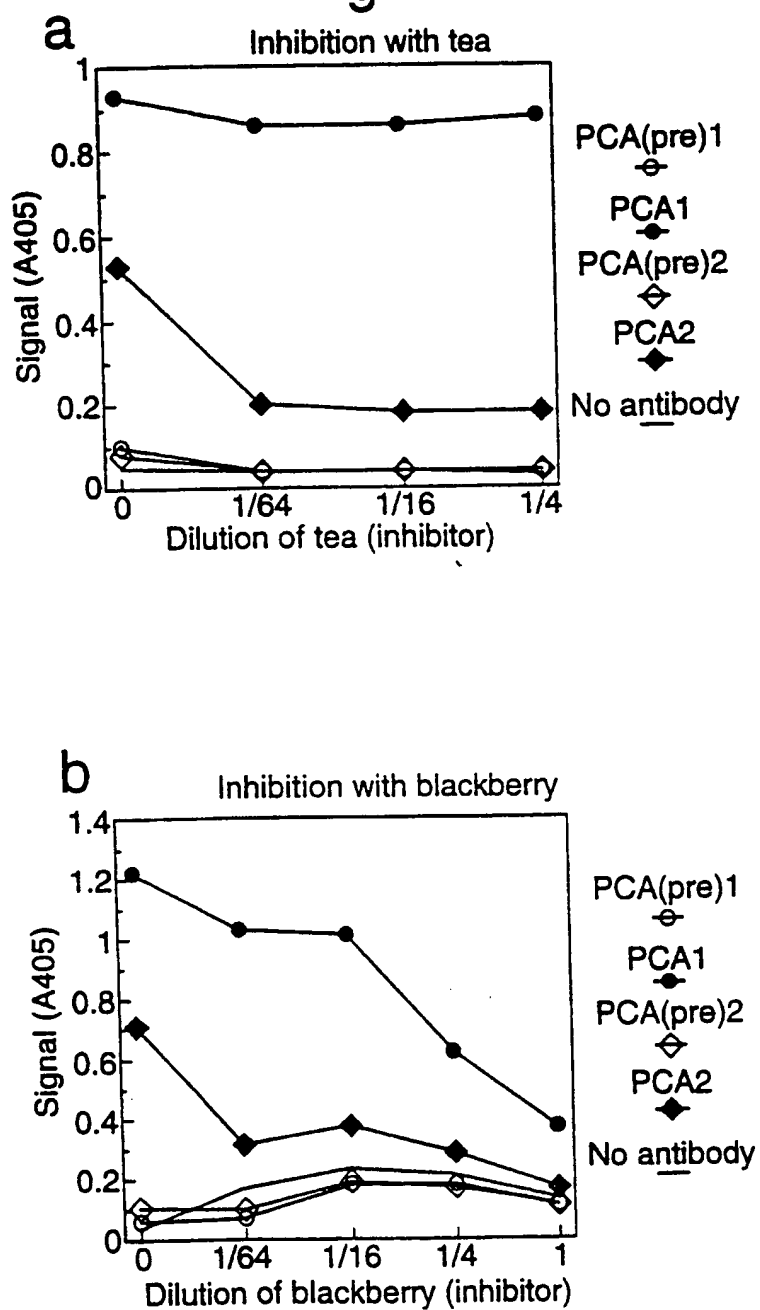


Fig.7.



(51) International Patent Classification <sup>6</sup> : <b>C11D 3/386, 3/384, 3/39, C12N 9/02, 9/08, C07K 16/46, A61K 39/00</b>		<b>A3</b>	(11) International Publication Number: <b>WO 98/56885</b> (43) International Publication Date: <b>17 December 1998 (17.12.98)</b>
(21) International Application Number: <b>PCT/EP98/03438</b> (22) International Filing Date: <b>29 May 1998 (29.05.98)</b> (30) Priority Data: 97304145.2                      13 June 1997 (13.06.97)                      EP (34) Countries for which the regional or international application was filed: <b>GB et al.</b> (71) Applicant (for all designated States except AU BB CA GB IE KE LK LS MN MW NZ SD SG SZ TT UG): <b>UNILEVER N.V. [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL).</b> (71) Applicant (for AU BB CA GB IE KE LK LS MN MW NZ SD SG SZ TT UG only): <b>UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4P 4BQ (GB).</b> (72) Inventors: <b>BEGGS, Thomas, Stewart; Unilever Research Colworth, Colworth House, Sharnbrook MK44 1LQ (GB). BERRY, Mark, John; Unilever Research Colworth, Colworth House, Sharnbrook MK44 1LQ (GB). DAVIS, Paul, James; Unilever Research Colworth, Colworth House, Sharnbrook MK44 1LQ (GB). FRENKEN, Leon, Gerardus, J.; Unilever Research Vlaardingen, Olivier van Noortlaan 120, NL-3133 AT Vlaardingen (NL). VERRIPS, Cornelis, Theodorus; Unilever Research Vlaardingen, Olivier van Noortlaan 120, NL-3133 AT Vlaardingen (NL).</b>		(74) Common Representative: <b>UNILEVER N.V.; Patent Division, P.O. Box 137, NL-3130 AC Vlaardingen (NL).</b> (81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b> <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: <b>25 March 1999 (25.03.99)</b>	
(54) Title: <b>BLEACHING ENZYMES</b> (57) Abstract <p>There is provided a bleaching enzyme capable of generating a bleaching chemical and having a high binding affinity for stains present on fabrics. Furthermore, there is provided an enzymatic bleaching composition comprising said bleaching enzyme and a surfactant and a process for bleaching stains present of fabrics.</p>			

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## INTERNATIONAL SEARCH REPORT

In International Application No

PCT/EP 98/03438

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C11D3/386 C11D3/384 C11D3/39 C12N9/02 C12N9/08  
 C07K16/46 A61K39/00

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## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C11D C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 540 784 A (PROCTER & GAMBLE) 12 May 1993	1-4, 9-14, 21, 24, 25 18, 19
Y	see page 3, line 31 see page 10, line 15 - line 26 see page 11, line 5 - line 25 see page 11, line 55 - line 57 ---	
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Y	see page 1, line 3 - line 10 see page 10, line 16 - line 29 ---	
-/-		

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

5 November 1998

Date of mailing of the international search report

11 02. 1999

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 98/03438

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 95 07972 A (UNILEVER) 23 March 1995  see page 1, line 4 - line 9 see page 1, line 19 - line 29 see page 10, line 35 - line 37 see page 13, line 14 - line 19; claim 1 ---	1-4, 21-25
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 98/03438

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
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2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

- 1) Claims :1-25 concerns the usage of enzymes for bleaching purposes
- 2) Claim : 26 concerns an immunogen of polyphenols and poly-L-proline, i.e.

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-25

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

CT/EP 98/03438

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